EXPERIMENT K-6-06

MORPHOMETRIC AND EM ANALYSES OF TIBIAL EPIPHYSEAL PLATES FROM COSMOS 1887 RATS

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SUMMARY

Light and electron microscopy studies were carried out on decalcified tibial epiphyseal plates of rats flown aboard Cosmos 1887 (12.5d flight plus 53.5h recovery). Analysis of variance showed that the proliferative zone of flight animals was significantly higher than that of synchronous controls, while the hypertrophic/calcification zone was significantly reduced. Flight animals had more cells than synchronous controls in the proliferative zone, and less in the hypertrophic/calcification region. The total number of cells, however, was significantly higher in flight animals. No differences were found for perimeter or shape factor of growth plates, but area was significantly lower in flight animals in comparison to synchronous controls. Collagen fibrils in flight animals were shorter and wider than in synchronous controls. The time required for a cell to cycle through the growth plate is 2-3 days, so most of the cells and matrix present were formed after the animals had returned to 1g, and probably represent stages of recovery from microgravity exposure, which in itself is an interesting question.

INTRODUCTION

The effect of spaceflight on bone development in growing rats has been studied using animals flown on the Soviet Cosmos series, and on Spacelab 3 (Morey-Holton and Arnaud, 1985). These studies have shown that bone formation ceases during spaceflight (Wronski and Morey, 1983), that matrix formed in flight does not mature normally, and consequently cannot mineralize normally (Simmons, et al., 1983) and that these changes are well underway after seven days of spaceflight (Russell and Simmons, 1985).

The cartilagenous epiphyses of the long bones of space rats have received less attention (only two studies have been conducted) even though some of the changes that are seen in bone --e.g. decreased length (Wronski, et al., 1981) and decreased trabecular bone mass (Je[illegible] et al., 1983) -- must originate in the epiphyseal region.

In the first study, epiphyses of rats flown aboard Cosmos 1129 (an 18.5 d flight) showed a delay in matrix vesicle production and mineralization, and lack of collagen maturation (Matthews, 1981).

In the other study, conducted in our laboratory, proximal tibial epiphyses of Spacelab 3 rats (7 d flight, 12 h recovery) were analyzed at the light and electron microscope levels using interactive image analysis and computerized planimetry, and cartilage differentiation was found to be markedly affected after only seven days of spaceflight (Duke, et al., 1985; Duke and Montufar-Solis, 1989). Heights of plates and zones of flight animals were less than those of controls, and there were fewer cells per column in the proliferative zone and the hypertrophic/calcification (H/C) zone. When both height of zones and cell number were considered, the greatest effect was on the proliferative zone. Areas of flight plates were significantly less than areas of control plates, and the shape factors of the two sets of plates were different. At the EM level, important alterations in matrix production and maturation were found (Duke and Montufar-Solis, 1989). Collagen fibers of flight animals were shorter and thinner than those of controls. Proteoglycan granules were found at higher densities (PGG/mm ^{2}) in flight animals. Areas of these granules were also measured and a significantly different pattern of zonal distribution of PGG sizes was found between the two groups (Duke and Montufar-Solis, 1989).

Some decrease in plate height on SL3 may have resulted from exposure to earth gravity during the twelve hour period between landing in California and sacrifice at Kennedy, especially since matrix organization was altered. To examine this question, a simulation of SL3 was carried out at Ames Research Center using the Holton suspended rat model (Morey, 1979). Besides testing the validity of the Holton model by allowing a direct comparison with SL3 animals, the sim also provided an

opportunity to determine what effect loading during the last 12 hours of SL3 may have had, since one group of animals was sacrificed at time of recovery (R+0) and another after an additional 12 hours of loading (R+12).

Growth plates analyzed in our laboratory showed that suspension of animals produced a significant decrease in plate height and in number of cells per column. An additional decrease was observed in both experimental and control groups during the final 12 hours of the experiment, but the differences were significant only in experimental animals. The percent reduction in plate height (17%) and cells/column (17%) were almost the same as those seen in SL3 (16% and 18%), but per zone measurements indicate different mechanisms might be involved in unloading due to spaceflight versus unloading due to suspension, since in suspended animals, the greatest effect was on the hypertrophic/calcification zone (Montufar-Solis and Duke, 1988).

These studies indicate that normal maturation and/or differentiation in the growth plate is altered under exposure to μg. From a detailed study of morphology, changes in matrix production and cellular proliferation due to exposure to μg can be elucidated, as can matrix calcification and matrix calcifiability.

The objectives of the present study were to look for differences in plate parameters (height, cell number, area, perimeter, shape factor), and cell and matrix ultrastructure, in proximal tibial epiphyseal plates of rats flown aboard the 12.5d Cosmos mission, and to compare any differences noted to those found in growth plates of SL3 and SL3-sim rats.

METHODS

Figure 1 shows a general outline of the complete experimental procedure.

Tissue Preparation

Decapitation of the animals was carried out by the Soviet team in Kazakhstan, 53.5 hours after landing of the 12.5 d flight. Using a fresh razor blade, the heads of right tibias were cut from the shaft and placed upside down on the dissecting surface. With another razor blade, the epiphysis was cut in half in the saggital plane (figure 2). The halves used in our studies were placed in screwtop Teflon vials containing cold (4 °C) fixative (2% paraformaldehyde in 0.1M cacodylate buffer with 0.5% glutaraldehyde, pH 7.4). The tissue was maintained at 4 °C for the entire 48 h fixation period. When samples arrived in Moscow, they were rinsed three times with 0.1M cacodylate buffer, and transferred into cold (4 °C) decalcifying solution (10% EDTA in 0.1M TrisHCl buffer, pH 7.4) for storage and shipment.

Samples (n=5/treatment) were received in our lab on November 4, 1987. Decalcification continued for 2 weeks with the EDTA solution being changed every other day. Following decalcification, the medial portion of the plate was embedded in Spurr for electron microscope studies, while the lateral portion was embedded in Paraplast for light microscopy (figure 2).

Light Microscopy and Image Analysis

Five micron coronal sections from the central region of the plate, alternately stained with hematoxylin and toluidine blue, were subjected to image analysis using the video input and digitizing components of the Bioquant system. Area, perimeter, and shape factor (4π area/perim ^{2}) were determined per section to obtain a mean value per animal. Following the definition of zones (figure 3) by Reinholt et al. (1984), determination of zone height and number of cells per zone were made using an ocular micrometer and magnification of 25X. Three measurements (left, middle, and right regions) were used to obtain a mean per section, and these

averaged to obtain a mean per animal. Statistical analyses were carried out using one-way ANOVA. Data was processed using the Number Cruncher Statistical Analysis System, version 3.1.

Electron Microscopy

After thick sections were taken for orientation, thin sections stained with uranyl acetate and lead citrate were examined in a Hitachi 11-E transmission electron microscope. A series of micrographs were taken in each zone, and measurements of collagen fibril length and width were made using a ZIDAS digitizing tablet. Means per section were averaged to obtain means per animal and statistical analyses were carried out using one-way ANOVA.

RESULTS

Light Microscopy

Sections of growth plates of each treatment are shown in figure 4. The difference in plate height between the basal group and the other three is due to aging of the animals. In the flight group, note the small, closely packed cells in the proliferative zone.

Results of all parameters measured in light microscopy and image analysis studies are summarized in table 1. The proliferative zone (PZ) of flight animals was significantly larger than that of synchronous controls (0.124mm vs 0.091mm) while the hypertrophic/calcification zone was significantly reduced (0.025mm vs 0.058mm-figure 5). These changes cancelled out so that the difference in total plate height (0.177mm vs 0.188mm) was not statistically significant.

The same pattern was seen in cell number per zone. Flight animals had more cells than synchronous controls in the proliferative zone (17.88 vs 12.87), and less in the H/C region (1.44 vs 3.74). The increase in cell number in the proliferative zone was so large that the decrease in the H/C zone did not compensate for it, resulting in a significantly higher number of cells in the plates of flight animals (19.31 vs 16.61-figure 6). The area of total growth plate was significantly lower in flight animals ($1mm^2$ vs $1.3mm^2$) than synchronous controls. No significant difference was observed in perimeter or shape factor between flight animals and synchronous controls.

Electron Microscopy

Pictures of collagen fibrils observed in each treatment are shown in figure 7. Collagen fibrils in flight animals were significantly wider than controls in the proliferative zone, (19.3 nm vs 14.9 nm) and also in the hypertrophic zone (18.7 nm vs 12.9 nm - figure 8). Flight animals showed a reduction in fibril length from controls in the proliferative zone, (905.5 nm vs 1212.7 nm) and in the hypertrophic zone (873.3 nm vs 943.1 nm-figure 9), but these differences are not significant due to small sample size and large variance in length measurements.

DISCUSSION

The lack of inflight animal sacrifice makes interpretation of data from flight studies difficult, especially so in the case of Cosmos 1887 where animals were at 1g for 53.5 hours prior to sacrifice (figure 10). Interpretation is also complicated by the responsiveness of growth plate to factors such as feeding time (Russell, et al., 1983), light/dark cycle (Simmons, 1974), and endocrine status (Russell, et al., 1984). Comparisons with SL3 and Holton's SL3 simulation are suspect as well, since the US flight and the simulation used a different rat strain, singly-housed animals, lower lipid food pressed into bars, and a 12-12 light/dark cycle. In spite of these

differences, several points of commonality were found between the three experiments (refer to Table 2).

Area of growth plates, measured with computerized planimetry, was less in each case, and, except for group SL3-sim/R+12, significantly less. In each case, the difference in area is probably a function of the decrease in height.

Perimeter was not significantly altered in any of the experiments, and shape factor (a function of area and perimeter^2) was significantly decreased in SL3 animals only. Since matrix organization was altered in these animals, the difference in shape factor could be due to compression effects on the growth plate during and after landing. Any such effect on the Cosmos growth plates would have been obliterated due to longer recovery time. Suspended animals, of course, are not freed from all gravitational effects during suspension and are not exposed to gravitational changes that occur during landing of spacecraft.

Examination of the data for heights of zones and number of cells leads to the speculation that during unloading (as in SL3-sim/R+0), cells and matrix accumulate in the reserve zone, and, upon reloading, receive stimuli causing them to move into the proliferative zone, thus depleting the reserve zone, as seen in SL3-sim/R+12, SL3 and Cosmos 1887.

The number of cells responding to a stimulus is dependent in part on the number of cells in the respondent population, and perhaps Cosmos 1887 had large numbers of cells in the reserve zone after 12.5d of spaceflight, which moved rapidly into the proliferation zone upon reloading. The large increase in proliferation zone is likely to be the beginning of the rebound phenomenon seen on previous Cosmos missions where tibial length of rats sacrificed 25d postflight was the same as or greater than controls (Yagodovsky et al., 1976; Holton and Baylink, 1978; Holton, et al., 1978, 1979).

This buildup and depletion in the reserve zone corresponds to the pattern seen in growth hormone production (Grindeland and Hymer, this volume). Release of growth hormone by cultured pituitary cells was found to be depressed after SL3, and somatotrophs implanted into hypophysectomized rats did not support tibial growth (Hymer, et al., 1985). Similar results were seen on the SL3-sim (Motter, et al., 1987). For Cosmos 1887, analysis of transport medium of flight cells showed increased growth hormone release, but after 6 days of culture, secretion dropped significantly (see Grindeland and Hymer, this volume).

The decrease in cell number in the H/C zone which was seen in each experimental case is likely due to lack of cell differentiation. The decreases seen in SL3 and both SL3-sim groups are less than that in Cosmos 1887, perhaps due to the latter's longer time in space. Of all the cell groups observed, the H/C zone is most likely comprised of cells that differentiate during the spaceflight period. This is so even for Cosmos 1887 with its long recovery time. The very large decrease seen here makes it likely that even after 53.5 hour of recovery, cells that proliferate postflight have yet to hypertrophy. If these proliferative cells hypertrophied and produced matrix, the height of the total plate would increase. Additional spaceflights and/or simulations are needed to answer questions regarding recovery.

Ultrastructural analyses on SL3 and Cosmos 1887 show that aggregation of collagen fibrils and of proteoglycan granules (PGG's) is altered by spaceflight. For SL3, collagen fibrils were shorter and thinner and proteoglycan granules, smaller and more numerous, than those of controls (Duke and Montufar- Solis, 1989). Measurements of PGG size and distribution in Cosmos 1887 growth plates are not yet complete, but preliminary observations indicate that PGG's of flight animals are bigger than those of synchronous controls. Changes in proteoglycan production produce concommitant changes in fibril aggregation (Duke, 1979), and it may be that the differences seen in

fibril size are secondary to differences in PGG production and/or aggregation. PGG's are also thought to be important in regulating mineralization. (Carrino, et al., 1985) and the impaired mineralization seen in flight animals may be due to defective proteoglycan production and/or processing.

A number of morphometric measurements have yet to be completed. Besides the size and density (number/unit area) of PGG's, the size, number and distribution of matrix vesicles, volumes of cells and matrix, and width of RER cisternae have not been determined, and are needed to complete the morphometric and ultrastructural picture of spaceflight effects upon rat growth plates. Additional experiments with very short recovery times, or, ideally, in-flight animal sacrifice, are needed to allow examination of effects of spaceflight uncomplicated by questions of post-flight exposure to 1g.

Nevertheless, there can be no doubt that spaceflight changes cell proliferation and differentiation, and matrix organization within the growth plate, and that these changes are reflected in decreased length and impaired mineralization of long bones.

ACKNOWLEDGMENTS

M. Campbell, R. Grindeland, V. Hubbard, E. Puente, C. Solis, C. Williams and Soviet Scientists.

REFERENCES

1. Carrino, D. A., M. Weitzhandler, and A. I. Caplan. 1985. Proteoglycans synthesized during the cartilage to bone transition. In: The Chemistry and Biology of Mineralized Tissues (W.T. Butler, ed.), pp. 197-208.

2. Duke, J. and W.A. Elmer. 1979. Effect of the brachypod mutation on early stages of chondrogenesis in mouse embryonic hind limbs: an ultrastructural analysis. Teratology 19:367-376.

3. Duke, J., L. Janer, J. Morrow, and M. Campbell. 1985. Microprobe analyses of epiphyseal plates from Spacelab 3 rats. The Physiologist 28.S217-S218.

4. Duke, J. and B. Montufar-Solis. 1989. Proteoglycan granules in growth plates of Spacelab 3 rats. In: Proceedings of the Third International Conference on the Chemistry and Biology of Mineralized Tissues. In press.

5. Holton, E. Morey and D.J. Baylink. 1978. K005: Quantitative analysis of selected bone parameters. In: Final Reports of US Experiments Flown on the Soviet Satellite Cosmos 782 (Rosenweig, S.M. and K.A. Souza, eds). (NASA TM-78525), pp. 321-351

6. Holton, E.M., R.T. Turner, and D.J. Baylink. 1979. K205: Quantitative Analysis of selected bone parameters. In: Final Report of U.S. Experiements Flown on the Soviet Satellite Cosmos 936 (Rosenweig, S.M. and K.A. Souza, eds). (NASA TM-78526), pp. 135-178.

7. Hymer, W.C., R. Grindeland, M. Farrington, T. Fast, C. Hayes, K. Motter and L. Patil. 1985. Microgravity associated changes in pituitary growth hormone (GH) cells prepared from rats flown on Spacelab 3. The Physiologist 28:S197-S198.

8. Jee, W.S.S , T.J. Wronski, E.R. Morey, and D.B. Kimmel. 1983. Effects of spaceflight on trabecular bone in rats. Am. J. Physiol. 244.(Regulatory Integrative Comp. Physiol. 13):R310-R314.

9. Matthews, J.L. 1981. Mineralization in the long bones. In: Final Reports of US Rat Experiments Flown on the Soviet Satellite Cosmos 1129, (Heinrich, M.R., and K.A. Souza, eds). NASA TM 81289, pp. 199-228.

10. Montufar-Solis, D. and J. Duke. 1988. Effect of simulated spaceflight on tibial epiphyseal plates. ASGSB Bulletin 1:34.

11. Morey-Holton, E.R. and S.B. Arnaud. 1985. Spaceflight and calcium metabolism. The Physiologist 28:S9-S12.

12. Morey, E.R. 1979. Spaceflight and bone turnover: correlation with a new model of weightlessness. Bioscience 29: 168-172

13. Motter, K., M. Vasques, C. Hayes, M. Kendall, G. Tietjen, W.C. Hymer and R. Grindeland. 1987. Effects of microgravity on rat pituitary growth hormone (GH) cell function: comparison of results of the SL-3 mission with rat "unloading" on Earth. Space Life Sciences Symposium: Three Decades of Life Science Research in Space, pp. 52-53.

14. Reinholt, F. P., A. Hjerpe, K. Jansson and B. Engfeldt. 1984. Stereological studies on the epiphyseal growth plate in low phosphate vitamin D- deficiency rickets with special reference to the distribution of matrix vesicles. Calcif. Tissue Int. 36:95-101.

15. Russell, J.E. and D.J. Simmons. 1985. Bone maturation in rats flown on the Spacelab 3 mission. The Physiologist 28:S235-S237.

16. Russell, J.E., W.U. Walker and D.J. Simmons. 1984. Adrenal/parathyroid regulation of DNA, collagen and protein synthesis in rat epiphyseal cartilage and bone. J. Endocr. 103:49-57.

17. Russell, J.E., D.J. Simmons, B. Huber and B.A. Roos. 1983. Meal timing as a Zeitgeber for skeletal deoxyribonucleic acid and collagen synthesis rhythms. Endrocrinology 113:2035-2042.

18. Simmons, D.J. 1974. Chronobiology of endochondral ossification. Chronobiologia 1:97-109.

19. Simmons, D.J., J.E. Russell, F. Winter, P. Tran Van, A. Vignery, R. Baron, G.D. Rosenberg and W.V. Walker. 1983. Effect of spaceflight on the non-weight bearing bones of the rat skeleton. Am. J. Physiol. 244 (Regulatory Integrative Comp. Physiol. 13):R319-R326.

20. Wronski, T.J. and E.R. Morey. 1983. Effect of spaceflight on periosteal bone formation in rats. Am. J. Physiol. 244(Regulatory Integrative Comp. Physiol. 13):R305-R309.

21. Wronski, T.J., E. Morey-Holton, C.E. Cann, C.D. Arnaud, D.J. Baylink, R.T. Turner, W.S.S. Jee. 1981. K305: Quantitative analysis of selected bone parameters. In: Final Reports of US Rat Experiments Flown on the Soviet Satellite Cosmos 1129, NASA TM 81289, pp. 101-125.

22. Yagodovsky, V.S., L.A. Triftanidi, and G.P. Gorokhova. 1976. Space flight effects on skeletal bones of rats (light and electron microscopic examination). Aviatio Space Environ. Med. 47:734-738.

TABLE 1. LIGHT MICROSCOPY AND IMAGE ANALYSIS RESULTS

GP Parameter	Basal (a)		Synchronous (b)		Vivarium (c)		Flight (d)	
Height/Zone (mm)								
Reserve	.053±	.002	.038±	.004a[2]	.039±	.002a[2]	.028±	.002a[1], c[2]
Proliferative	.104	.005	.091	.032	.091	.006	.124	.004a[3],b[1],c[2]
Hyper/Calcif	.112	.009	.058	.001a[1]	.064	.004a[1]	.025	.004a[1],b[1],c[1]
Total GP	.269	.010	.188	.006a[1]	.194	.007a[1]	.177	.008a[1]
Cell Number/Zone								
Proliferative	15.39	1.00	12.87	.45	2.91	.85	17.88	.57 b[1],c[1]
Hyper/Calcif	6.30	0.36	3.74	.15 a[1]	3.68	.15 a[1]	1.44	.23 a[1],b[1],c[1]
Total GP	21.69	0.95	16.61	.44 a[1]	16.60	.90 a[2]	19.31	.53 b2,c2
Area (mm^2)	1.4	0.11	1.3	.09	1.2	.07	1.0	.016a[1],b[2]
Perimeter (mm)	14.27	1.73	14.31	.55	12.76	.39b[4]	13.11	.23
Shape Factor	.095	0.011	.08	.004	.09	.003	.074	.0029c[2]

Mean ± S.E.M. for each treatment, and level of significant difference between groups. Comparisons made between all groups. Lower case letters states group to which the comparison was made, and the number next to it indicates level of significance:

1 - $p \leq .001$
2 - $p \leq .01$
3 - $p \leq .03$
4 - $p \leq .05$

TABLE 2. CHANGES OBSERVED IN COSMOS 1887, SL3, AND SL3-SIM

Parameter	SL3-SIM		SL3	CM 1887
	R+0	R+12		
Area	- 16.6*	- 11.2 %	- 9 %*	- 23 %*
Perimeter	- 5.2	+ 3.2 %	+ 1.8 %	- 8 %
Shape Factor	- 7.5%	- 7.3 %	- 12.5 %*	- 7 %
Height RZ	+ 44.5%*	- 10 %	- 11 %*	- 26 %
PZ	+ 1 %	- 7 %	- 14 %*	+ 36 %*
H/C Z	- 26 %*	- 25 %*	- 19 %*	- 57 %*
Total Growth Plate	- 12 %	- 17 %*	- 16 %*	- 6 %
Cell number PZ	- 6 %	- 16 %	- 21 %*	+39 %*
H/C Z	-			
23 %	- 20 %*	- 15 %	- 61 %*	
Total Growth Plate	- 14 %*	- 17 %*	- 18 %*	+ 16 %*

* = significantly different; $p \leq .05$
- = decrease; + = increase from correspondent controls.

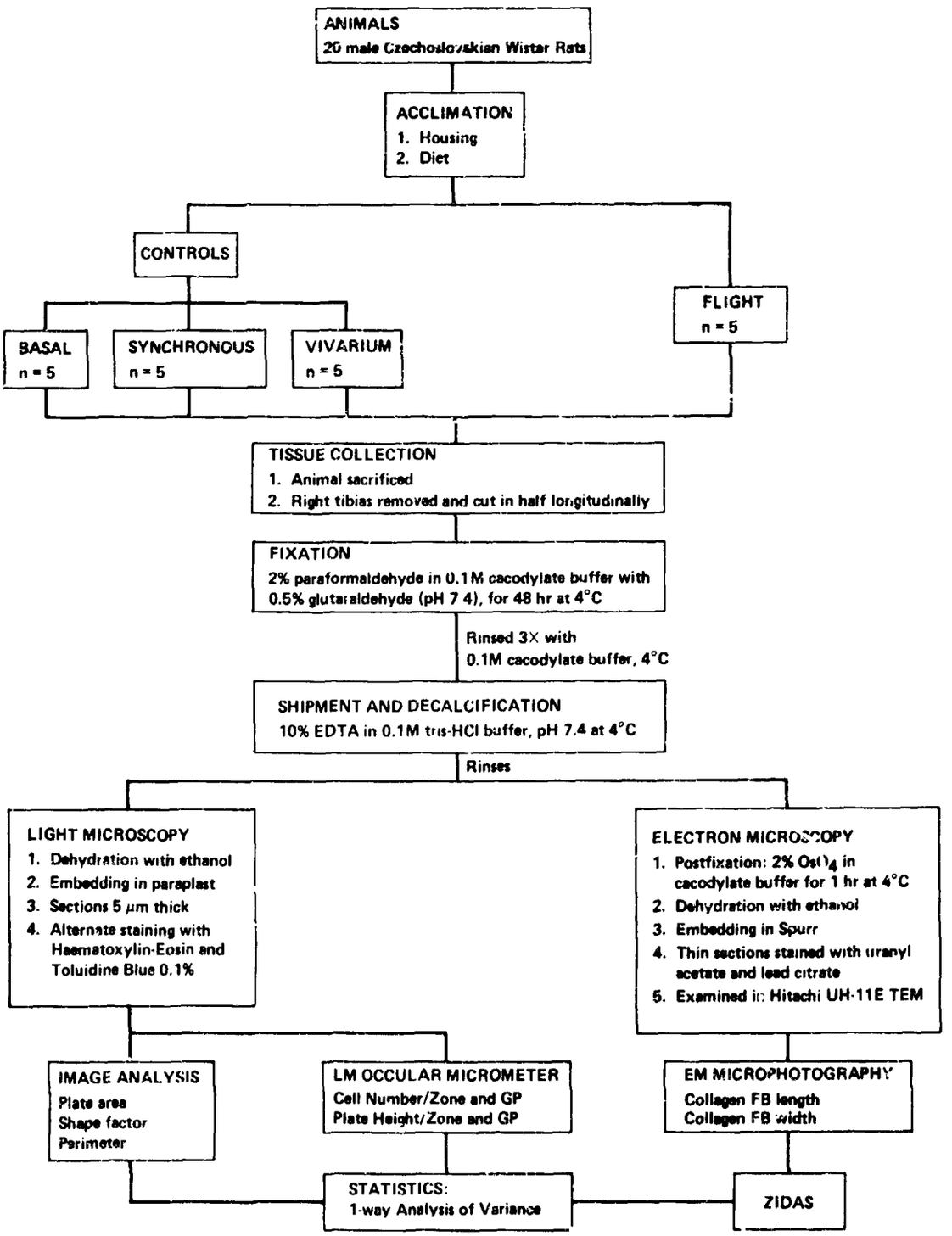


Figure 1. Flow chart of the general procedure followed in Experiment K-6-06: Morphometric and EM analyses of tibial epiphyseal plates from Cosmos 1887 rats.

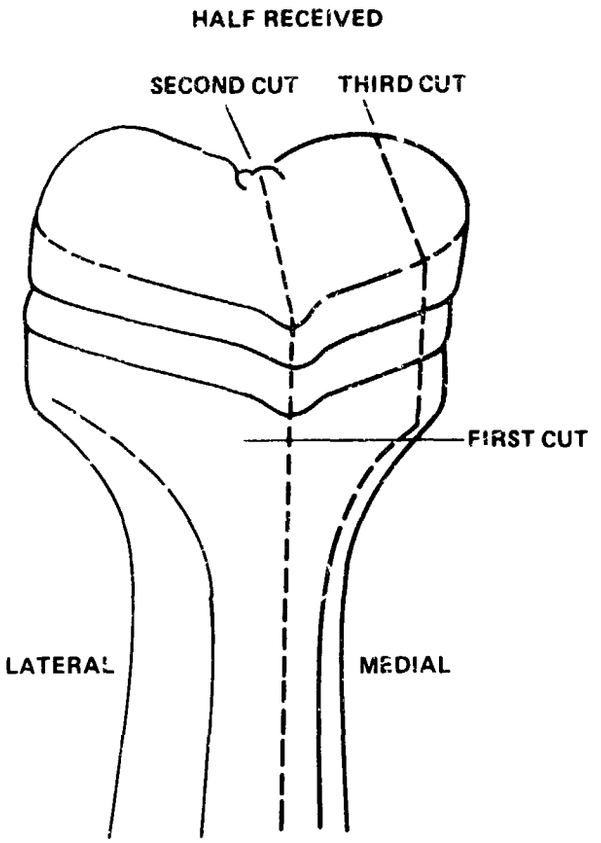
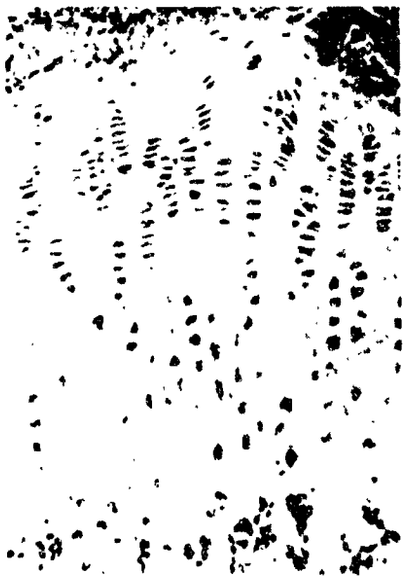


Figure 2. Dissection of right tibias.



*Taken from Reinholt et al., 1984.

Resting: from upper plate border to top of first cell column.

Proliferative: ratio cell width/ height > 2.

Hypertrophic: ratio cell width/ height < 2.

Calcification: not distinguishable in decalcified sections.

Figure 3. Definitions of zones of growth plate.

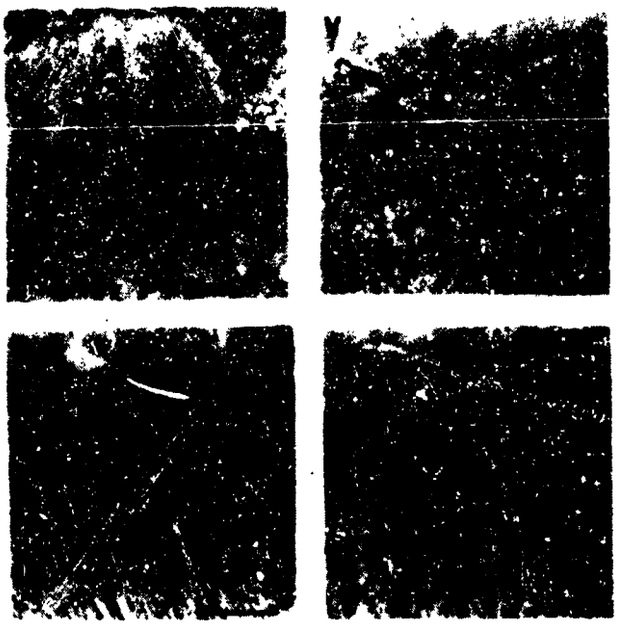


Figure 4. Representative sections of growth plates from each group.

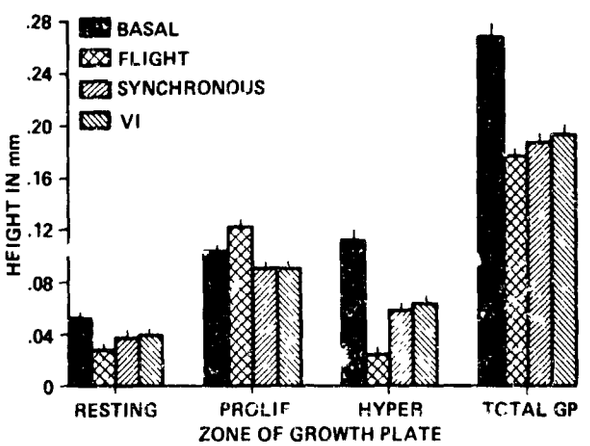


Figure 5. Mean height per zone and total growth plate. For detailed comparison between groups, refer to Table 1.

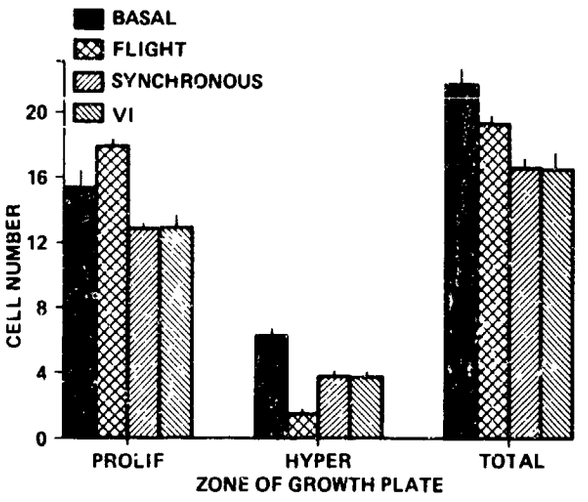


Figure 6. Mean cell number per zone and total growth plate. For detailed comparison between groups, refer to Table 1.

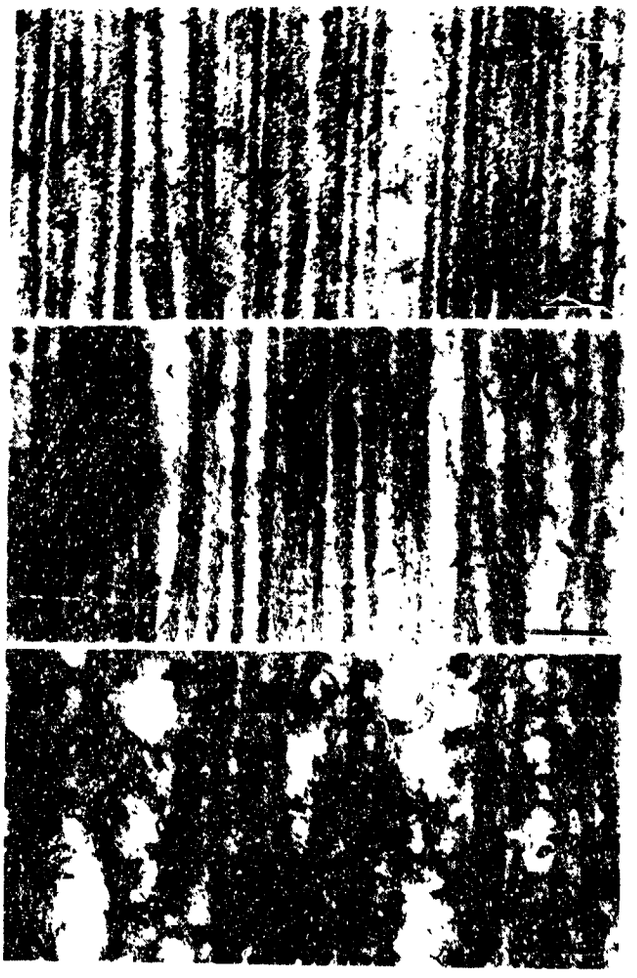


Figure 7. Representative collagen fibrils from each group.

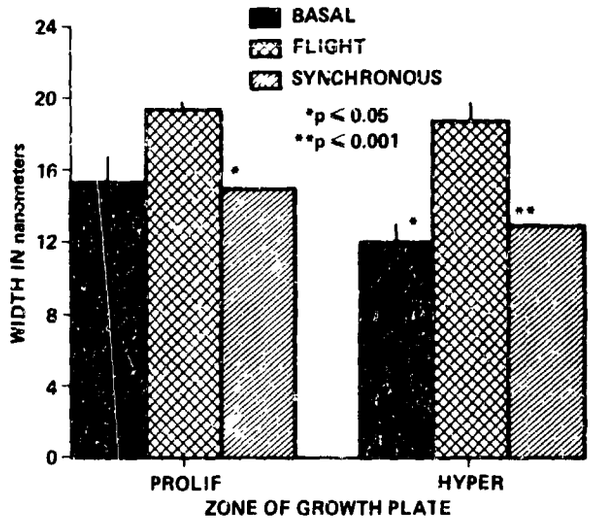


Figure 8. Mean collagen fibrial width per zone of growth plate.

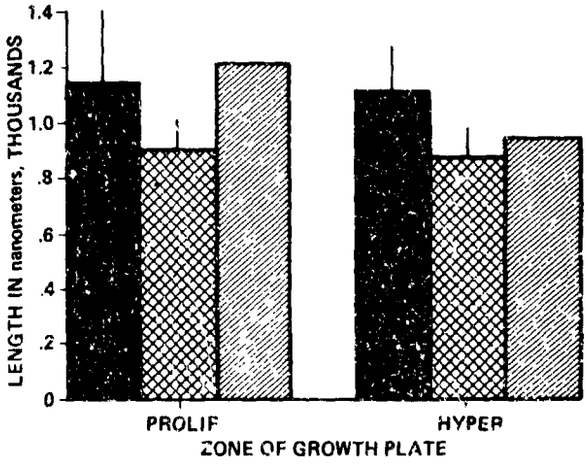


Figure 9. Mean collagen fibrial length per zone of growth plate.

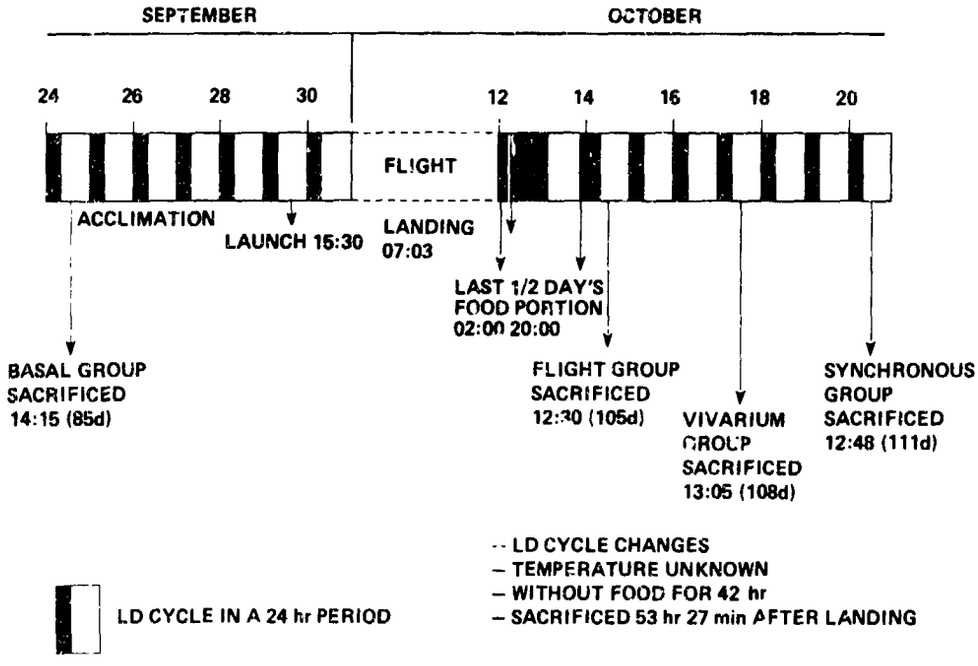


Figure 10. Time line for Cosmos 1887.